Exhibit 23

VETERINARY ANALYTICAL TOXICOLOGY

Enzymatic–Spectrophotometric Method for Determination of Cholinesterase Activity in Whole Blood: Collaborative Study

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Ten collaborating laboratories assayed 4 blind duplicate pairs of whole bovine blood for cholinesterase activity. The 4 sample pairs ranged from normal (100%) to severely organophosphorus-inhibited (<10%) activity. Collaborators also received commercially available human lyophilized serum as an external control and a chromate solution to evaluate spectrophotometer performance. The Ellman kinetic assay was performed on a 1:1000 dilution of the whole blood in pH 8.0 phosphate buffer. The method monitors the increase in absorbance at 412 nm caused by formation of 5-thio-2-nitrobenzoic acid (yellow reaction product). Repeatability standard deviations (RSD_r) ranged from 4.30 to 14.2%; reproducibility standard deviations (RSD_R) ranged from 6.99 to 19.3%. The lower limit of detection was estimated to be 0.10 μmole/mL/min. The method has been approved interim official first action by AOAC.

At sufficient doses, all organophosphorus insecticides have a common mechanism of acute toxic action, the inhibition of acetylcholinesterase (AChE) and pseudocholinesterase (PChE) (1–3). (The Enzyme Commission names and numbers are acetylcholine acetylhydrolase [EC 3.1.1.7] and acylcholine acylhydrolase [EC 3.1.1.8], respectively.) The measurement of either or both of AChE and PChE has been shown to be a sensitive method for monitoring exposure to cholinesterase inhibitors such as organophosphorus insecticides, which are irreversible inhibitors, and carbamate insecticides, which are generally considered reversible inhibitors (2, 3).

AChE, also known as true or RBC cholinesterase, and PChE, or nonspecific cholinesterase, are found in varying proportions in the blood of domestic and laboratory animals and wildlife (2, 4, 5). Specific substrates can be utilized for these cholinesterase forms; however, in clinical veterinary and environmental work, a rapid screening method is desired in order to assess an animal's probable exposure to organophosphorus insecticides. If whole blood is assayed, the combined influence of AChE and PChE is monitored. The term cholinesterase (ChE) will be used here to denote the combined AChE and PChE contributions of the sample.

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The recommendation has been approved interim official first action by the General Referee, the Committee Statistician, the Committee on Feeds, Fertilizers, and Related Topics, and the Chairman of the Official Methods Board, and will be recommended for adoption official first action at the 104th AOAC Annual International Meeting, September 9–13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* 74, January/February issue.

Collaborative Study

Kinetic procedures that allow continuous measurement of the reaction rate provide easy detection of enzyme activity and monitoring to ensure that linear kinetics are observed. The kinetic method of Ellman (6) which utilizes acetylthiocholine as substrate and a 1:1000 dilution of whole blood with pH 8.0 phosphate buffer, was used for the collaborative study. The proposed method was submitted to 10 participating laboratories along with 4 blind duplicate pairs of bovine whole blood samples, a commercially available human serum sample to serve as an external control, and a chromate solution to evaluate spectrophotometer performance. The laboratories were instructed to refrigerate all samples and reagents on arrival and to perform the assay within 72 h.

Samples for the collaborative study were prepared as follows: Approximately 1000 mL whole blood (anticoagulant, Ca-EDTA) was collected from clinically normal cattle. The blood was mixed and separated into 4 groups. One group remained untreated, and 3 groups were incubated with varying amounts of dilute paraoxon solution to achieve enzyme activity near targeted levels (75, 55, and <10% of normal). The 4 groups were monitored periodically for 2 weeks before submission to the collaborating laboratories to ensure that levels were stable. Twenty aliquots of about 7 mL each were removed from each group and placed in screw-cap polypropylene vials. The 20 vials were separated into 2 groups of 10 each to serve as blind duplicate pairs.

The SeraChem® control serum used in the assay has established values for a Boehringer Mannheim Diagnostics cholinesterase kit of 1.42 ± 0.45 μmole/mL/min (corrected to 25°C) based on the Ellman reaction; however, a different pH (7.2) and reagent strengths are utilized. In addition, a wavelength of 405 nm is specified to easily conform to instrumentation used for human clinical samples. The associate referee has used this product with the proposed assay for over 5 years and has established an expected range with the product. The lot number used for the study (148-056) was assayed 55 times from January 29, 1988, through June 10, 1988, and a mean and SD of 1.62 and 0.13 μmole/mL/min, respectively, were established with the proposed method. A 2-SD range of 1.36–1.88 could be considered acceptable; however, the authors prefer to use the range of 1.41–1.73 established by this study as the acceptable range.

The lyophilized human serum control was prepared for distribution to collaborators by pooling the contents of 5 vials

Table 1. Collaborative data for enzymatic determination of cholinesterase activity in bovine whole blood[a]

	Group 1		Group 2		Group 3		Group 4	
Lab.	1	4	2	8	3	6	5	7
1	2.03	1.84	1.12	1.25	1.53	1.77	0.31	0.24
2	2.12	2.19	1.27	1.28	1.63	1.75	0.32	0.37
3	1.97	1.92	1.07	1.06	1.40	1.41	0.24	0.19
4	2.09	1.95	1.21	1.22	1.53	1.51	0.28	0.29
5	2.38	2.15	1.49[b]	1.12	1.59	1.30	0.21	0.21
6[c]	1.40	1.40	0.84	0.83	1.01	1.09	0.15	0.19
7	2.19	2.24	1.32	1.21	1.82	1.96	0.28	0.31
8	2.06	1.92	1.12	1.19	1.42	1.45	0.18	0.29
9	1.94	1.43	1.12	0.67[b]	1.46	0.18[b]	0.22	0.26
10	2.13	2.05	1.24	1.29	1.69	1.55	0.26	0.25

[a] Units are μmole/mL/min. Each group is a blind duplicate pair.
[b] Value was deleted from data set by Dixon's outlier procedure and was not included in statistical analysis.
[c] Eliminated by rank sum test.

= path length, cm; and DF = dilution factor = 1000.

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Results and Discussion

Results were received from 10 collaborating laboratories (Table 1). Laboratory 5 reported excessive noise when samples 1, 2, 3, and 4 were run; however, only sample 2 was eliminated by the Dixon test. Laboratory 5 also reported sporadic problems with the spectrophotometer before and after the collaborative study, and the light source required replacement within 3 weeks after the study. Laboratory 6 was eliminated by the rank sum test. Laboratory 9 had a widely divergent result in one sample from group 3 and one from group 2, which were eliminated by the Dixon test. Statistical analysis was performed on all remaining samples according to the *Statistical Manual of the AOAC* (8). These results are presented in Table 2.

All laboratories were requested to record operating temperature, instrument type, spectral band width, and results from the serum control and spectrophotometer check solution. All laboratories used a double beam spectrophotometer except Laboratories 4 and 7, who used single beam instruments. Table 3 is a summary of temperatures, control values, and spectrophotometer performance results reported by collaborators. Two laboratories did not report the spectral band width of their instruments. The 8 remaining laboratories reported band widths from 0.6 to 3 nm, with no observable effect on the results. For every sample assayed, the A value at each time point was recorded by the collaborators. These data were used to fit a linear regression line for A vs time. The correlation coefficients ranged from 1.00 to 0.87 for the 80 data sets. All but 2 data sets had correlation coefficients from 1.00 to 0.95.

Table 3. Summary of interlaboratory variables in collaborative study on enzymatic determination of cholinesterase activity in bovine whole blood[a]

Statistic	Temp. °C	Control serum, μmole/mL/min	Spectrophotometer performance, A units
Mean	23.2	1.62	0.195
SD	1.00	0.10	0.007
Range	22.0–25.5	1.41–1.73	0.186–0.210

[a] Values from Laboratory 6, which was eliminated by the rank sum test, are not included here.

The proposed method employed ambient temperatures because temperature-controlled cells are not always available in diagnostic laboratories. The temperature data for participating laboratories (with the exception of Laboratory 6, which was eliminated by the rank sum test) are reported in Table 3. No effect on the enzyme activity results was observed over a temperature range of 22–25.5°C. In the authors' opinion, temperature control at 25°C is recommended when possible to maintain the best control over the reaction rate; however, results for the 9 laboratories which allowed all reagents to reach ambient temperatures of 22–25.5°C were acceptable.

Previous research has shown that the minimum limit of detection is about 0.10 μmole/mL/min, which assumes that 0.0014 A/min change can be accurately determined. The exact limit is determined by the noise level of the instrument used and is not of importance in this study.

Clinical interpretation is based on comparison of the individual value to the normal population for that species. The groups evaluated for this study were targeted at 100, 55, 75, and <10% of normal activity for the bovine. The laboratory means for the 4 groups resulted in 100, 58, 78, and 13% for groups 1–4, respectively.

The collaborators generally offered no comments regarding the method and had few problems.

Recommendation

The Associate Referee recommends that this method be adopted official first action.

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Table 2. Statistical data from collaborative study on enzymatic determination of cholinesterase activity in bovine whole blood[a]

	Group 1	Group 2	Group 3	Group 4
Statistic	1, 4	2, 8	3, 6	5, 7
Mean, μmole/mL/min	2.03	1.17	1.58	0.26
Repeatability standard deviation, s_r	0.15	0.05	0.11	0.04
Relative standard deviation, RSD_r, %	7.37	4.30	7.01	14.2
Reproducibility standard deviation, s_R	0.20	0.08	0.18	0.05
Relative standard deviation, RSD_R, %	10.1	6.99	11.5	19.3

[a] Dixon outliers and laboratory eliminated by rank sum test excluded.

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Flame Atomic Absorption Spectrometric Determination of Serum Zinc: Collaborative Study

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A method for determining serum zinc concentrations was collaboratively studied by 9 laboratories. Serum samples in polypropylene tubes are directly diluted with 0.03% Brij 35, and zinc is determined by flame atomic absorption spectrophotometry (AAS). A certified standard reference material serves as an external control. Repeatability relative standard deviations (RSD_r) were 1.2 and 11.4% and reproducibility relative standard deviations (RSD_R) were 6.1 and 12.9% for serum zinc concentrations of 6.36 and 0.63 μg/mL, respectively. The method has been approved interim official first action by AOAC.

Zinc is an essential element for the normal growth, reproduction, and life expectancy of animals (1). Under normal conditions, serum zinc concentrations are maintained relatively constant. However, serum concentrations may be higher after ingestion of zinc ointments or zinc-containing foreign objects (2, 3), and reduced concentrations have been reported in response to stress or disease (4–6). Since the normal range for serum zinc varies widely between and within species (0.6–2.7 ppm), a reliable, standardized method is needed in order to meaningfully evaluate changes in levels of serum zinc.

Over the past 20 years, various atomic absorption spectrophotometric (AAS) methods have been reported in the literature. An early method (7), which requires deproteinization of samples prior to analysis, is time consuming and samples are subject to contamination. These concerns are addressed in the majority of current flame AAS methods, which specify plastic apparatus where possible and minimize sample manipulation by performing the analysis directly after various dilution regimens (8–10). While intralaboratory precision of these methods is good, no collaborative study data are available. Also, none of these methods specifies background correction. Results from this laboratory show that without background correction, determinations average 15% higher compared with those in which readings are corrected.

Although serum zinc determination is not technically difficult, validity of the results depends on avoiding contamination from ambient zinc. We found that preparing serum samples for atomic absorption spectrophotometry (AAS) by using standard acid digestion techniques increased zinc concentrations 5–10 times over expected normal values. We attributed this increase to sample contamination from glassware, reagents, and/or environmental sources. Therefore, we developed a method in which sample handling is kept to a minimum and only disposable plasticware contacts the sample, which greatly reduces the possibility of contamination.

In the method, serum is diluted 1 + 5 with a 0.03% solution

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